

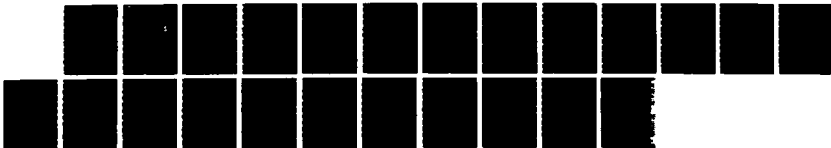
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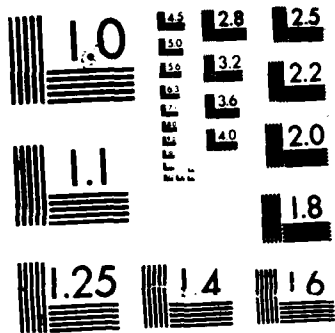
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SCREENING OF IMMUNOENHANCING DRUGS WITH ANTIVIRAL  
ACTIVITY AGAINST MEMBERS OF THE ARENA-, ALPHA-,  
AND ADENOVIRIDAE

Annual Report

February 1, 1987

Paul A. LeBlanc and Alvin L. Winters

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# ABSTRACT

A series of 13 immunomodulatory compounds or mixtures of compounds have been screened for antiviral activity with either Venezuelan equine encephalitis virus, guinea pig adapted Pichinde virus, or a lung tropic plaque purified variant of mouse adenovirus as the challenge virus. Only Bordetella pertussis vaccine (BPV) and a Boivin extract of Bordetella pertussis (EP-LPS) were found to give consistent protection against a virus. The protective effect of both was seen only against adenovirus. BPV treatment appeared to result in the prolongation of life of guinea pigs challenged with Pichinde as compared to controls. CL246,738 treated mice showed a prolongation of life over buffer treated controls when challenged with Venezuelan equine encephalitis virus. The effect of EP-LPS on the immune status of injected animals was investigated in an attempt to identify the critical change that resulted in protection. EP-LPS injection did not result in any dramatic changes in the immunologic status of the treated animal. However, there was a slight decrease in the number of antibody producing cells found in the spleen after treated animal were immunized with sheep red blood cells. Further, EP-LPS treated animals showed an increase in natural killer cell activity in the spleen as compared to either buffer or E. coli LPS treated controls.

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## FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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## INTRODUCTION

This project was designed to test a series of immunomodulatory compounds designated by the U.S. Army Research and development Command for their ability to protect animals from an otherwise lethal infection of virus. The three animal - virus models used in the study represented a viral pneumonia (mouse adenovirus in the mouse), encephalitis (Venezuelan equine encephalitis in the mouse), and hemorrhagic fever (Pichinde virus in the strain 13 guinea pig). Due to cost, the two systems which utilized mice were used as screening systems. If a positive result was obtained in one of the mouse systems, the guinea pig - Pichinde model was tested.

Once a modulator was found to be able to protect an animal against a viral infection, the second level of testing was an evaluation of the changes in the immune response induced by the modulator. A standard set of immunological tests were used to determine the changes induces in the T, B, NK, macrophage functions. We have previously shown that the Boivin extract of Bordetella pertussis (EP-LPS) was able to protect against lethal adenovirus infections (1). This was used as a positive control in the adenovirus system and as a starting point in studies intended to determine the crucial compartment of the immune response, which was stimulated to give protection.

## Materials and Methods

### Viruses.

Venezuelan equine encephalitis (VEE) strain 68U201 (2) was obtained from Dr. Peter Jahrling, USAMRIID, and was propagated in primary chicken embryo cell culture to prepare a working stock. Pichinde virus strains were obtained from the same source. The Pichinde strains were both the parental (3739) and guinea pig lethal (GP-11) strains (3, 4). Each strain was propagated separately in Vero cell cultures to prepare a working stock of virus. Lung tropic, plaque purified strain of mouse adenovirus (MadV) was derived by one of the investigators (ALW) and has been previously reported (5). Working stocks of MadV were prepared in roller cultures of L929 cells.

### Endotoxin Detection.

All reagents were tested for the presence of bacterial endotoxin by the gel formation, Lymulus amebocyte lysate (LAL) test (6). The LAL was obtained from Associates of Cape Cod (Woods Hole, MA) with a 0.03 endotoxin unit per ml sensitivity. The sensitivity was confirmed with each test by the titration of standard lipopolysaccharide (LPS). Materials that test negative in this test were defined as endotoxin free.

### Cells and Cell Culture.

Modified Eagles' minimal essential medium (HMEM) was prepared from a powdered mix (Auto-POW MEM, Flow Laboratories, McLean, VA) and supplemented with glutamine (2 mM, Flow), sodium bicarbonate (2 mg/ml, Sigma Chemical Co., St. Louis, MO), and HEPES (15 mM, Sigma). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories, Logan, UT. Clone 929 of L-cells (CCL 1) were obtained from the ATCC, Rockville, Maryland, and were propagated in HMEM + 10% FBS. Vero (African green monkey kidney) cells were obtained from Dr. Gillespie (University of North Carolina Cancer Center, Chapel

Hill, NC) and propagated in HMEM +10% FBS. Chicken embryo cell (CEC) were produced by trypsinization of 9-11 day old chicken embryos according to the method of Scherer (7) except that HMEM + 10% FBS was used as growth medium. The preparation of bone marrow culture derived macrophages has been previously been described (8).

#### In Vivo Tests (Level 1).

A compound was administered as recommended by the source (dosages, times, and routes are indicated in the relevant tables) and then the mice were challenged with either 400 pfu of the 68U201 strain of VEE, subcutaneously;  $>2 \times 10^7$  pfu of Mouse adenovirus pt4, intraperitoneally. Guinea pigs were challenged with 40 pfu of guinea pig-adapted Pichinde virus, subcutaneously.

#### Functional Assays (Level 2).

When evaluating cellular activity the cells were harvested or the animal immunized at the same time after modulator treatment as viral administration in successful level 1 testing. This was done to ensure that the cells obtained were in the same state as when the virus had been administered. Likewise the immune system of the intact animal should be in the same state when challenged with test antigens as when challenged with virus.

The number of antibody producing cells in the spleens of mice was evaluated by the Mishell and Dutton (9) modification of the Jerne and Nordin plaque assay (10). Mice were immunized with 0.01 ml of a 20 % v/v sheep red blood cell (SRBC) suspension per gram of body weight. Plaque forming units were enumerated 3 and 4 days after SRBC injection.

Cytotoxic T-cell activity was determined by immunizing C3H/HeN mice ( $H-2^k$ ) with  $10^7$  P815 cells ( $H-2^d$ ) at times determined as above. Seven to twelve days later the spleens were removed and tested for T-cell mediated cellular cytotoxicity in a  $^{51}\text{Cr}$ -release assay using labelled P815 cells as targets as described (11).

The ability of a compound to act either as a trigger for previously primed macrophages or to directly activate unprimed macrophages was determined using a  $^{51}\text{Cr}$ -release assay as previously described (12). A supernatant from a concanavalin A stimulated spleen culture was used as a source of gamma interferon (13). The ability of a compound to stimulate macrophages to produce interferon was determined by exposure of cultures of day 10 bone marrow culture derived macrophages to various concentrations of the compound for two hours. The stimulus was then removed and the cells reincubated in fresh medium for an additional 24 hours. At that time, the supernatants were collected and interferon levels determined by a micro-plaque reduction assay as previously described (14).

## RESULTS and DISCUSSION

A series of 13 compounds or combinations of compounds have been screened for antiviral activity with either VEE, guinea pig adapted Pichinde virus, or mouse adenovirus as the challenge virus. The results of those tests are given in Tables 1-8.

Those tables indicate that only BPV or EP-LPS gave consistent protection against a virus challenge. The block polymers have given protection against MadV in one experiment. These experiments are being repeated. However, the only protective effect for any agent was seen only

with mouse adenovirus challenge. It is not known if there would have been any protection against mouse adenovirus of those compound only tested against VEE. There was insufficient material to test many of the preparations against all viruses. Lederle compound CL246,738 did not prevent death from VEE challenge. However when prolongation of life is determined, mice treated with a single oral dose of this compound had significantly better survival than controls (see Table 9). We are presently investigating multiple dose regimens and different administration protocols to determine if this prolongation of life can be extended to survival. This material is also being tested in the adenovirus system. Using 6 guinea pigs per group, BPV gave a mean survival time of 16.2 days vs 13.8 days for the diluent control. This prolongation of life was significant when tested by the Wilcoxon 2 sample rank test at a p value of <0.01.

Since only BPV and EP-LPS gave consistent protection against a viral infection, only these agents were candidates for second level screening as defined in our research protocol. However, the biochemical nature of EP-LPS is much better defined and there are many biological effects of BPV that have been shown not to be relative to protection from virus infection (15, 16). Therefore, EP-LPS was selected over BPV for screening of general immunomodulatory activity. The object of these screens was to define the general changes in host defense activity induced by EP-LPS. It was hoped that one particular component of the host defense would be stimulated. If this had happened it would indicated that a particular component of the host response was important to the antiviral activity of EP-LPS. In fact, there was not a overwhelming increase in the activity of any of the components of the host response tested ( see Tables 10-15). In some experiments extracts of E. coli LPS are included. This was done because these LPS preparations do not have the antiviral activity of the Boivin extract of Bordetella pertussis. Thus we are looking for a difference not only between buffer controls and EP-LPS injected animals but between EP-LPS and other LPS injected animals.

The first impression from these results is that there is not any one major change in the immune status of the animals treated with EP-LPS as compared to controls of either buffer or other LPS preparations. There are slight changes that may be suggestive of a rearrangement of the immune system. There was a decrease in the number of direct plaque forming cells when expressed at the per cell level. However, this was mitigated to some extent due to the increased number of cells found in the spleens of mice treated with EP-LPS and SRBC as compared to those injected with buffer and SRBC. Compared to these decreases in B-cell activity. There was no change detectable in the anti-allotypic cytotoxic T-cell response of mice treated with either EP-LPS or control buffer either on the per cell or the per spleen basis. There were increases in the splenic natural killer cell activity of EP-LPS treated mice when compared to either buffer controls or E. coli LPS treated mice. This increase in activity was noted both on a per cell basis and on a per spleen basis. Macrophages, another nonspecific cytotoxic cell, elicited by injection of EP-LPS were not found to be increased in activity or sensitivity to activating stimuli above those elicited by buffer. Further, EP-LPS was not more efficient than E. coli LPS at directly activating macrophages in vitro or as serving as a trigger signal after interferon priming of macrophage monolayers. Finally, EP-LPS was able to induce production of interferon from macrophage cultures at a level comparable to that of E. coli LPS. Thus the overall effect of EP-LPS

treatment seems to be a slight decrease in the B-cell response to an antigen and an increase in NK cell activity.

One interesting results occurred when testing for the amount of prostaglandin E produced by bone marrow culture derived macrophages after stimulation with different LPS preparations. The experiment did not work for its intended purpose because the control macrophages were inactive in the cytolytic assay. This was due to the contamination of the medium used to grow the bone marrow cultures with "wild" endotoxin from the Nalgene filter units used in preparing the medium. When medium used to grow macrophages was split into two samples, one filtered and one not filtered, the macrophages grown in the filtered medium failed to respond in the assay while those grown in unfiltered and the endotoxin free medium did respond (data not shown). In the face of these nonresponding macrophages, only EP-LPS was able to induce cytolytic activity (Table 16). This would suggest that EP-LPS acts through a second process to activate macrophages and that it is different from that used by other LPS's. This avenue has not been pursued because we have not been able to reproducibly "create endotoxin contaminated" medium by the addition of known amounts of purified LPS.

We have attempted to produce a mouse adapted strain of Pichinde virus according to the method of Jahrling et al. (4). To that end we have injected weanling C3H/HeN mice with Pichinde virus, harvested their spleens 7 days later, and reinjected new mice with that splenic homogenate. This procedure has not generated a mouse lethal strain of Pichinde after nine serial passages. In fact, the homogenate isolated from the first mouse did not contain any detectable virus. To investigate this problem, mice were injected with Pichinde virus and sacrificed at various times after infection and assayed for the presence of virus. No virus was found at any time point tested. We have also looked for plaque type variants in the parental strain population. No apparent plaque variants are visible within the parental population. We have therefore adopted two new strategies. The first has been to inject neonatal mice and the second has been to establish a mixed culture system of Vero and L929 cells. Neonatal mice have been infected with  $1 \times 10^5$  pfu of parental strain Pichinde virus. After five days, the livers of infected mice were collected and a 10 % w/v suspension was made by grinding the livers in a mortar and pestle with HMEM + 10% FBS as a diluent. This material was titered in a plaque assay on Vero cells and injected into a second group of mice. Virus was isolated from the livers of these mice at a concentration of  $1.8 \times 10^5$  pfu / ml of the 10 % suspension. After several passages in neonatal mice, we will gradually increase the age of the mice infected with the virus in order to obtain an adult mouse lethal strain. The mix cell culture system has not yet yielded results. The hope is that the mixed cell bed will maximize the chances of selecting a variant that can grow in mouse cells (L929).

Treatment	Challenge Virus	Time to Challenge	<u># Dead</u> <u># Infected</u>
Bestatin, 4 mg/kg	MadV	7 days	6/10
Diluent	MadV	7 days	6/10
Bestatin, 4 mg/kg	MadV	7 days	8/10
Diluent	MadV	7 days	9/10

Table 1. Assessment of antiviral activity of bestatin. Bestatin (Sigma, St. Louis, MO) were administered ip seven days before ip challenge with  $>2 \times 10^7$  pfu of mouse adenovirus.

Treatment	Challenge Virus	Time to Challenge	<u># Dead</u> <u># Infected</u>
Exp. 1			
EP-LPS, 2 mg/kg	MadV	7 days	0/10
EP-LPS, 2 mg/kg	VEE	7 Days	10/10
Diluent	VEE	7 days	10/10
Exp. 2			
EP-LPS, 0.8 mg/kg	MadV	7 days	3/10
EP-LPS, 0.08 mg/kg	MadV	7 days	10/10
Diluent	MadV	7 days	10/10

Table 2. Assessment of the antiviral activity of Boivin extract of Bordetella pertussis (EP-LPS). EP-LPS was administered ip seven days before ip challenge with  $>2 \times 10^7$  pfu of mouse adenovirus, or subcutaneous challenge with 400 pfu of 68U201 strain of VEE virus. Diluent was LAL<sup>-</sup> PBS.

Treatment	Challenge Virus	Time to Challenge	# Dead # Infected
BPV, 10 mg/kg	MadV	7 days	0/10
BPV, 0.4 mg/kg	MadV	7 days	9/19
Diluent	MadV	7 days	15/20
BPV, 10 mg/kg	VEE	7 days	10/10
Diluent	VEE	7 days	10/10
BPV, 10 mg/kg	Pichinde	7 days	6/6
Diluent	Pichinde	7 days	6/6

Table 3. Assessment of the antiviral activity of Bordetella pertussis vaccine<sub>7</sub> (BPV). BPV was administered ip seven days before ip challenge with  $>2 \times 10^7$  pfu of mouse adenovirus, subcutaneous challenge with 400 pfu of 68U201 strain of VEE virus, or subcutaneous challenge with 40 pfu of Pichinde virus. Diluent was 0.02% sodium methiolate.

Treatment	Challenge Virus	Time to Challenge	# Dead # Infected
GBA, 80 ug/kg	MadV	7 days	10/10
GBA, 8 ug/kg	MadV	7 days	10/10
Diluent	MadV	7 days	10/10

Table 4. Assessment of the antiviral activity of gliding bacteria adjuvant (GBA). GBA, a polysaccharide extract of Cytophaga, was obtained from Dr. William Usinger, Univ. Calif., Berkley. GBA was administered ip seven days before ip challenge with  $>2 \times 10^7$  pfu of mouse adenovirus.

Treatment	Challenge Virus	Time to Challenge	# Dead # Infected
Exp 1			
Control	VEE	24 hrs	3/3
CL246,738, 100 mg/kg	VEE	24 hrs	4/4
Exp 2			
Control	VEE	24 hrs	9/9
CL246,738, 100 mg/kg	VEE	24 hrs	10/10

Table 5. Assessment of the antiviral activity of Lederle compound CL246,738. Mice were treated with orally administered CL246,738 (100 mg/kg) in 0.5 ml of pyrogen free PBS or 0.5 ml of PBS alone. 24 hours later mice were challenged with 400 pfu of 68U201 strain of Venezuelan equine encephalitis virus subcutaneously.

Treatment	Challenge Virus	Time to Challenge	# Dead # Infected
Exp 1			
Control	VEE	8 hrs	5/5
TNF, 100 ug/kg	VEE	8 hrs	10/10
Control	MadV	8 hrs	5/5
TNF, 100 ug/kg	MadV	8 hrs	6/10
Exp 2			
Control	MadV	8 hrs	10/10
TNF, 200 ug/kg	MadV	8 hrs	10/10

Table 6. Assessment of the antiviral activity of tumor necrosis factor (TNF). Original recombinant tumor necrosis factor was received as a powder from Dr. Bruce Butler (Howard Hughes Medical Institute, Univ. Texas, Dallas, TX) and was reconstituted in 1 ml of pyrogen free water. Part was diluted to 10 ug/ml for use in experiment 1, the remainder was frozen at -70° for use in experiment 2. Mice were injected intravenously. Eight hours later the mice were challenge by virus ( $>2 \times 10^7$  pfu mouse adenovirus, pt4, ip: 400 pfu strain 68U201 of VEE, sc).

Treatment	Challenge Virus	Time to Challenge	# Dead # Infected
50 ug TDM + 25 ug MPL	VEE	7 day	10/10
100 ug TDM + 50 ug MPL	VEE	7 days	10/10
250 ug TDM + 100 ug MPL	VEE	7 days	10/10
25 ug WS + 50 ug TDM + 25 ug MPL	VEE	7 days	10/10
50 ug WS + 100 ug TDM + 50 ug MPL	VEE	7 days	10/10
100 ug WS + 100 ug TDM + 100 ug MPL	VEE	7 days	10/10
100 ug TDM	VEE	7 days	9/9
250 ug TDM	VEE	7 days	10/10
500 ug TDM	VEE	7 days	10/10
25 ug STM	VEE	7 days	9/9
50 ug STM	VEE	7 days	10/10
100 ug STM	VEE	7 days	10/10
100 ug PA-PE	VEE	7 days	9/9
400 ug PA-PE	VEE	7 days	10/10
1200 ug PA-PE	VEE	7 days	10/10
100 ug S-209	VEE	7 days	19/10
500 ug S-209	VEE	7 days	9/9
1500 ug S-209	VEE	7 days	10/10
Control	VEE	7 days	29/29

Table 7. Assessment of the antiviral activity of combinations of Ribi immunomodulators. A series of combinations of Ribi immunomodulators (trehalose diamycolate, TDM; monophosphoryl lipid A, MPL; Bacillus-Calmettje-Guerin cell wall skeleton, WS; Salmonella typhimurium mitogen, STM; pyridine extract of Propionibacterium acnes, PA-PE; and compound S-209) were prepared by the manufacturer as dried material. Following manufacturer's directions these were reconstituted and administered by intraperitoneal injection seven days prior to the subcutaneous injection of 400 pfu of strain 68U201 of VEE. Control was pyrogen free PBS.



Treatment	Challenge Virus	Time to Challenge	# Dead # Infected
PBS	MadV	7 days	9/10
Block	MadV	7 days	0/10
EP-LPS, 400ug/kg	MadV	7 days	10/10
Block + EP-LPS	MadV	7 days	0/10

Table 8. Assessment of the antiviral activity of block polymers. Mice were injected intraperitoneally with EP-LPS in pyrogen free PBS, or a mixture of block polymers consisting of 1.25 mg of both L121 and T150R1 with or without 10 ug EP-LPS emulsified in PBS with 0.2 % Tween 80 and 10 % Drakeol mineral oil for a final concentration of 400 ug/kg EP-LPS, 50 mg/kg of each of the polymers. Seven days later, the mice were challenged with an intraperitoneal injection of  $>2 \times 10^7$  pfu of mouse adenovirus (pt 4).

Days post virus	Cumulative deaths										Dead infected	
	4	5	6	7	8	9	10	11	12	13		14
Treatment												
Exp 1												
Control	0	0	0	1	2	3						3/3
CL246,738	0	0	0	0	0	1	2	2	3	3	4	4/4
Exp 2												
Control	2	4	9									9/9
CL246,738	0	1	6	8	9							10/10

TABLE 9. Ability of compound CL246,738 to prolong life after a normally lethal viral infections of Venezuelan equine encephalitis virus. Mice were treated with orally administered CL246,738 (100 mg/kg) in 0.5 ml of pyrogen free PBS or 0.5 ml of PBS alone. 24 hours later mice were challenged with 400 pfu of 68U201 strain of Venezuelan equine encephalitis virus subcutaneously. Experiment 1 was too small for statistical evaluation. For experiment 2, the prolongation of life was significant at  $0.05 < p < 0.01$  by Wilcoxon two sample rank test.

	N	Treatment	Day	plaques/ $10^6$ cells	Plaques/spleen ( $\times 10^3$ )
Exp. 1	1				
	2	PBS	3	132.5	10.7
	2	EP-LPS	3	47.5	7.6
	3	PBS	4	714. $\pm$ 293	88. $\pm$ 34
	3	EP-LPS	4	350. $\pm$ 138	109. $\pm$ 45
Exp. 2					
	4	PBS	4	317. $\pm$ 170	62. $\pm$ 30
	4	EP-LPS	4	53. $\pm$ 35	16. $\pm$ 10

Table 10. Modulation of B-cell activity by EP-LPS. Mice were inoculated intraperitoneally with 0.5 ml of either EP-LPS (100 ug/ml) in endotoxin free PBS or with 0.5 ml of endotoxin free PBS 7 days before immunization with 0.2 ml of a 10% (V/V) SRBC suspension by the intraperitoneal route. Mice were sacrificed at either day 3 or 4 after inoculation of the SRBC suspension. Spleens were removed individually and a direct Jerne plaque assay was performed using the slide modification of Mishell and Dutton. Number of plaques were determined as the mean number of plaques per  $1 \times 10^6$  cell from 4 slides: 2 plated at  $10^5$  cells per slide and 2 plated at  $5 \times 10^4$  cells per slide. Total number of nucleated cells were determined by hemacytometer counts and the plaques per spleen calculated.

Treatment	Cells/spleen ( $\times 10^6$ )	Specific Release		Cytotoxic unit/spleen ( $\times 10^6$ )
		5:1	80:1	
PBS	8.8 $\pm$ 2.8	29.8 $\pm$ 6.99	86.0 $\pm$ 14.6	6.48 $\pm$ 4.45
EP-LPS	14.0 $\pm$ 4.0	26.6 $\pm$ 12.5	81.8 $\pm$ 18.9	7.86 $\pm$ 2.30

Table 11. Modulation of T-cell activity by EP-LPS. Mice (3/group) were inoculated intraperitoneally with 0.5 ml of either EP-LPS (100 ug/ml) in endotoxin free PBS or with 0.5 ml of endotoxin free PBS 7 days before immunization with  $10^7$  P815 mastocytoma cells. Mice were sacrificed 12 days after immunization and the cells recovered from each spleen individually. Cell number was determined by hemacytometer counts.  $^{51}\text{Cr}$ -labelled P815 target cells were incubated with varying concentrations of spleen cells to yield different effector to target ratios. E:T ratios of 5:1 and 80:1 are shown here. Specific release was calculated in triplicate from release of experimental wells - spontaneous release  $\pm$  freeze/thaw - spontaneous release. One cytotoxic unit represents the number of effector cells per target to yield 50% specific release. Results are the mean  $\pm$  standard deviation of groups of 3 mice.

Treatment	Cells/spleen (x10 <sup>6</sup> )	Specific Release		Cytotoxic unit/spleen (x10 <sup>5</sup> )
		50:1	200:1	
Medium	8.3±1.1	31.7±8.6	49.1±9.0	7.9±4.4
EP-LPS	7.0±0.8	38.1±6.9	61.3±5.5	10.5±3.6

Table 12. Modulation of natural killer cell activity by EP-LPS. Mice (4/group) were inoculated intraperitoneally with 0.5 ml of either EP-LPS (100 ug/ml) in endotoxin free medium or with 0.5 ml of endotoxin free medium 7 days before mice were sacrificed and the spleen cells isolated. The cells were recovered from each spleen individually. Cell numbers were determined by hemacytometer counts. <sup>51</sup>Cr-labelled YAC-1 target cells were incubated with varying concentrations of spleen cells to yield different effector to target ratios. E:T ratios of 50:1 and 200:1 are shown here. Specific release was calculated in triplicate from release of experimental wells - spontaneous release ÷ freeze/thaw - spontaneous release. One cytotoxic unit represents the number of effector cells per target to yield 50% specific release. The numbers of cells recovered per spleen were determined by hemacytometer counts. Results are the mean ± standard deviation of groups of 4 mice.

Treatment	Cells/spleen (x10 <sup>6</sup> )	Specific Release		Cytotoxic unit/spleen (x10 <sup>5</sup> )
		12.5:1	100:1	
Medium	6.5	2.1	10.5	8.9
Medium	6.6	3.5	13.5	10.7
EP-LPS	9.4	4.6	15.3	18.1
EP-LPS	8.5	10.6	17.4	40.5
E. coli	11.3	1.5	8.3	7.3
E. coli	11.7	1.6	7.3	7.7

Table 13. Comparison of the modulation of NK activity by EP-LPS and phenol extracted E. coli LPS. Mice were inoculated intraperitoneally with 0.5 ml of either EP-LPS (100 ug/ml) in endotoxin free medium, phenol extracted 0111:B4 E. coli LPS (100 ug/ml) in endotoxin free medium, or with 0.5 ml of endotoxin free medium 7 days before mice were sacrificed and the spleen cells isolated. The cells were recovered from each spleen individually. Cell numbers were determined by hemacytometer counts. <sup>51</sup>Cr-labelled YAC-1 target cells were incubated with varying concentrations of spleen cells to yield different effector to target ratios. E:T ratios of 12.5:1 and 100:1 are shown here. Specific release was calculated in triplicate from release of experimental wells - spontaneous release ÷ freeze/thaw - spontaneous release. Data represent the results of individual mice. One cytotoxic unit represents the number of effector cells per target to yield 50% of maximum specific release for this experiment. All of the titrations reached maximums of less than 20% specific release.

<u>Treatment</u>	<u>Interferon</u>	<u>Percent Specific Release</u>
EP-LPS (ng/ml)		
2	-	-4.4
1	-	0.9
0.5	-	3.1
0.25	-	5.2
0.125	-	2.0
0.06	-	-1.0
2.0	+	13.7
1.0	+	-5.7
0.5	+	-8.7
0.25	+	-6.8
0.125	+	-4.4
0.06	+	-3.9
<u>E. coli LPS</u> (ng/ml)		
2	-	5.52
1	-	1.6
0.5	-	2.5
0.25	-	2.2
0.125	-	5.0
0.06	-	8.1
2.0	+	67.7
1.0	+	64.3
0.5	+	32.6
0.25	+	-0.3
0.125	+	-4.1
0.06	+	-4.0

Table 14. Ability of EP-LPS to activate macrophages for cytolytic activity. Macrophages derived from 10 day cultures of murine bone marrow cells grown in the presence of L-cell conditioned medium as a source of CSF-1 were used as effectors. P815, mastocytoma cells were labelled with  $^{51}\text{Cr}$  and used as targets at a single effector to target ratio (2.5:1). Macrophage monolayers were exposed to stimulated for 4 hours before the addition of target cells. Macrophages were exposed to varying concentrations of EP-LPS from Bordetella pertussis or phenol extracted lipopolysaccharide from E. coli 0111:B4 either in the presence or the absence of 0.4 IRU/ml of gamma interferon (a supernatant from a 72 hour culture of Con A stimulated spleen cells). Each condition was run in triplicate. Specific release was calculated using the mean of the triplicates in the following formula:

$$\text{specific release} = \frac{\text{experimental} - \text{spontaneous release}}{\text{freeze/thaw} - \text{spontaneous release}}$$

Concentration (ng/ml)	Interferon Titer of Supernatants from Macrophage Cultures Treated With:		
	EP-LPS	LPS	ECB
Exp. 1			
100	> 8	4	< 2
10	4	4	< 2
1	4	< 2	< 2
0.1	< 2	< 2	< 2
Exp. 2			
100	> 8	> 8	< 2
10	> 8	> 8	< 2
1	< 2	< 2	< 2
0.1	< 2	< 2	< 2

Table 15. Comparison of the levels of interferon induced in macrophage cultures by different endotoxin extracts. Titer is given in units. One unit is defined as that amount of interferon sufficient to inhibit by 50 % the formation of plaques by a standard dose of VSV. EP-LPS is a Boivin extract of Bordetella pertussis. LPS is the lipid A rich fraction of a phenol extraction of E. coli O111:B4, provided by Dr. David Morrison. ECB is a Boivin extract of E. coli. Each value represents three separate cultures assayed in triplicate.

Pretreatment of macrophages		Concentration of Supernatant Used	Specific <sup>51</sup> Cr-Release	
			Exp 1	Exp 2
EP-LPS	100 ng/ml	1:2	70±6	24±3.5
		1:4	63±7	18±1
		1:8	61±4	11±3
		1:16	49±1	6±2
ECB	100 ng/ml	1:2	15±12	3±2
		1:4	8±6	2±1
		1:8	8±7	3±2
		1:16	8±1	0±1
LPS	100 ng/ml	1:2	19±2	4±2
		1:4	5±2	3±1
		1:8	4±3	4±1
		1:16	3±2	1±1
Medium		1:2	14±18	2±1
		1:4	7±12	2±1
		1:8	11±15	2±0
		1:16	9±15	1±1

Table 16. Activation of macrophages by supernatants from EP-LPS treated macrophage cultures. Macrophage cultures were exposed to medium containing the indicated concentrations of EP-LPS, ECB, or LPS. Control cultures received medium alone. Each condition was done in triplicate. After a 2 hr incubation period, supernatants from each culture were harvested separately and filtered. After supernatants in a were harvested, dilution of each were prepared and added to fresh macrophage monolayers. Each dilution was tested in triplicate. The final concentration of supernatant in the well is indicated in this well. The medium in each well also contained a final concentration of  $1 \times 10^{-6}$  M indomethacin and 100 ng/ml LPS. Cultures were then incubated for 24 hrs before the addition of target cells. After 24 hr incubation with supernatants, macrophage monolayers were washed and <sup>51</sup>Cr-labelled P815 mastocytoma cells were added. After an addition 16 hr of incubation half the supernatant was removed from each culture and the released radioactivity determined. Specific release was determined by the formula:

$$\text{specific release} = \frac{\text{experimental} - \text{spontaneous release}}{\text{freeze/thaw} - \text{spontaneous release}} \times 100$$

EP-LPS is a Boivin extract of Bordetella pertussis. ECB is a Boivin extract of E. coli. LPS is the lipid A rich fraction of a phenol extraction of E. coli 0111:B4, provided by Dr. David Morrison.

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